Enzymatic synthesis of analogs of bacterial lipid A and design of biologically active LPS-antagonists and -mimetics

M. Bulusu, J. Hildebrandt, C. Lam, E. Liehl, H. Loibner, I. Macher, D. Scholz, E. Schütze, P. Stütz, H. Vyplel, and F. Unger

Sandoz Forschungsinstitut G.m.b.H., Brunnerstr. 59, A-1235 Vienna, Austria

Abstract: *Lipid A, the lipid anchor of lipopolysaccharide (LPS) to the outer membrane of Gram-negative bacteria may be regarded as the most potent immunostimulatory but highly endotoxic substance. In a search for clinically useful substructures of lipid A, we synthesized analogs of lipid X, the reducing monosaccharide of lipid A, and of UDP-Lipid X as potential substrates for bacterial lipid A synthase, and obtained acylated glucosamine-(1-->6)-disaccharide-1-phosphates in good yield. The LPS-mimetic monosaccharide SDZ MRL 953 appears to represent the minimum substructure of lipid A with immuno-stimulatory properties of potential clinical relevance.*

Introduction

Lipid A has the capacity in animals to protect against lethal doses of endotoxin by induction of so called early-phase tolerance to LPS and also to protect against bacterial and fungal model infections. However, at higher concentration it exerts lethal endotoxicity. Fig. 1 shows the structure of lipid A from *E. coli* and some *Salmonella* strains as an example.

Fig. 1

Myr = myristyl
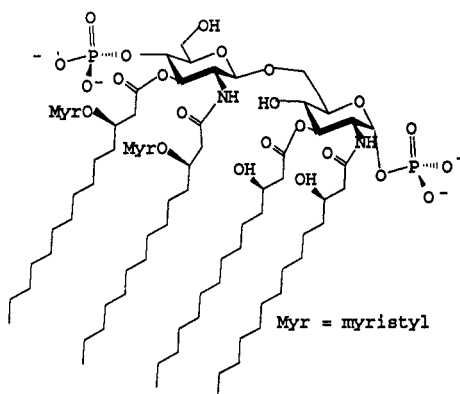
Some years ago, our group started a synthetic program for analogs of lipid A that would, hopefully, maintain the potentially beneficial immunostimulatory effects such as increased resistance to infections, but exhibit less endotoxicity. More selective biological activities were to be expected from new partial structure analogs of lipid A.

Our synthetic program consisted of two main steps:
* Synthesis of monosaccharide analogs of lipid X (3,4,6,7,9), the N,O-acyclated glucosamine-1α-phosphate as reducing end of lipid A

* Reaction of these components with UDP-lipid X in the presence of a preparation of lipid A synthase from an overproducing strain of *E. coli* (7,8,9,12,14).
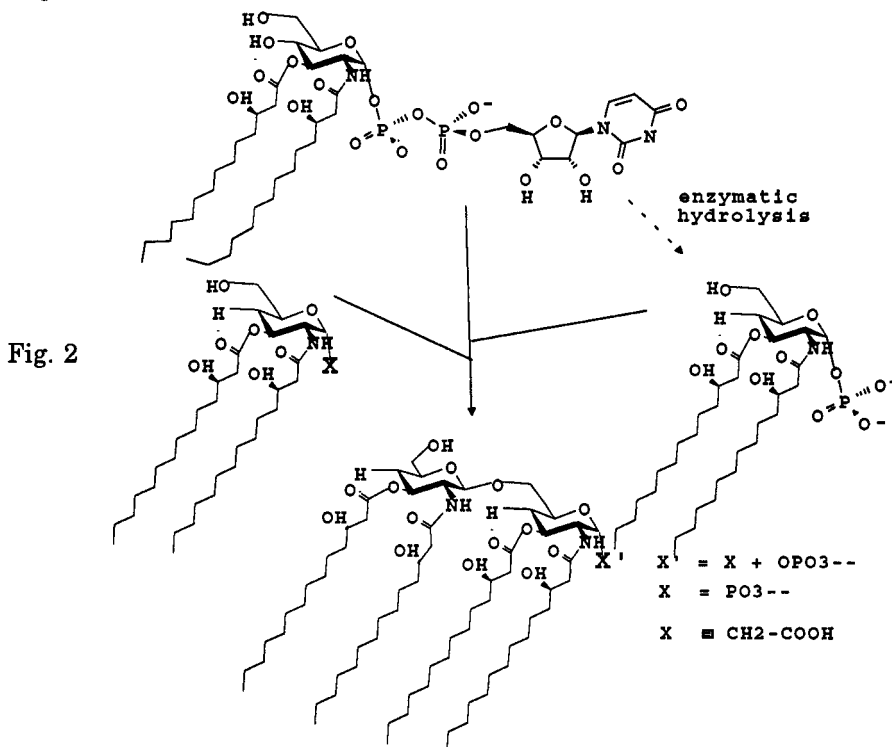
The synthesis of lipid X has been described by us and others (3,4). We were the first to show that highly purified lipid X is totally devoid of immunostimulatory effects, a controversial issue in the previous literature. It was found that small amounts of disaccharides as by-products from the last synthetic step can indeed change the biological profile of lipid X (5).
After having established the analytical tools for reliably purifying these amphipathic glucosamine mono- and disaccharides, a wide range of analogs of lipid X was synthesized. The following less obvious modifications deserve to be mentioned here:

* Replacement of the anomeric O-phosphate by the metabolically stable "isosteric" 1-α-phosphonate (6) or 1-α-C-glycosidic acetic acid (7, 8)
* Introduction of an additional (unnatural) 3-(R)-hydroxy-myristic acid moiety or a (unnatural) phosphate group into position 4 (7)
* Replacement of the acyclamido group of lipid X in position 2 by a fluoro group (9)

These compounds were allowed to react in the presence of UDP-lipid X (10) with a crude lipid A synthase preparation from an E.coli strain (MC 1061/ p5r8 Δ2515) as constructed by Raetz et al. (11), which overproduced this enzyme 1000-fold. In most instances, the expected new glucosamine (1-->6)-disaccharide analogs were formed in good yield and with reasonable time kinetics (7). Purification of end products was more difficult, but proceeded analogously to the lipid X case described above.
While a UDP-lipid X carrying an additional phosphate group at position 4 did not react at all (in accordance with the established biosynthetic pathways for lipid A), incorporation of the 1-phosphonate analog of lipid X was possible but required 3-5 days (12). Purification of this reaction mixture was particularly difficult due to solubility problems and contamination with the natural lipid A precursor 3 as competitive product. This sequence of events is summarized below:


Fig. 2

X' = X + OPO3 --
X = PO3 --
X = CH2-COOH

General Procedure for the Enzymatic Synthesis of Acylated Glucosamine Disaccharides

The preparation of lipid A synthase after fermentation of the overproducing *E. coli* strain has been described in detail (7). With substrate analogs structurally closely related to lipid A, a complete turnover of reactants was generally observed when 1.1 equivalent of UDP-lipid X was allowed to react in a tris(hydroxymethyl)-aminomethane containing buffer medium at pH 7.0. Reaction kinetics were conveniently checked by TLC or RP-HPLC. If a particular reaction was not complete overnight, another portion of enzyme was added to the reaction mixture, or work-up was started. Generally, the reaction rate could be accelerated by the addition of up to 15% of methanol, ethanol, or dimethylformamide .

## Syntheses of C-glycosidic or Fluorinated Substrate Analogs

The α- and β-C-glycosidic "bioisosteric" analogs of lipid X were obtained by a Wittig reaction of a suitably protected glucosamine derivative *1* (7). As could be expected, only the α-isomer was accepted as substrate analog by the enzyme. The essential steps of this synthesis are shown in fig. 3. The reaction conditions could be controlled such that the acyclic Wittig product *2* was isolated a single trans-isomer (30% yield), or as a mixture of α/β isomers *3* in the presence of DBU.

Fig. 3
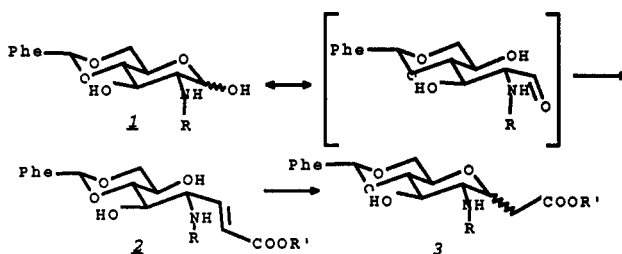
The critical step for the preparation of the 1-phosphonate analog of lipid X as substrate analog to the enzymatic synthesis consisted in the treatment of the α-D-gluco-configured trichloroacetimidate *4* with P(OMe)$_3$ as key reaction (6). The azidophosphonate *5* could be obtained in 76% yield.

Fig. 4
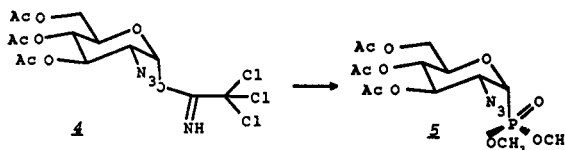
Access to the 2-deacylamino-2-fluoro-analog of lipid X was possible through a reaction of the glycan *6* with acetyl hypofluorite in glacial acetic acid as key step, leading to a 7:1 mixture of gluco- vs. manno-configured products *7* and *8* in a total yield of 70 % (13).

Fig. 5

X = F, Y = H   *7*

X = H, Y = F   *8*
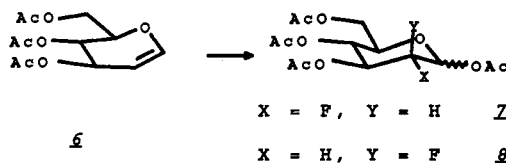
SDZ MRL 953 Selected for Clinical Trials.

Roughly 250 compounds originating from the above synthetic strategies were subjected to an immunopharmacological screening, in order to identify a useful candidate for clinical testing.

The monosaccharide SDZ MRL 953, **9**, and the disaccharide SDZ 880.924, **10**, were found to fulfill these requirements of selective immunopharmacological activities best (8). For practical reasons, **9** was ultimately selected for further development. Its preclinical biological properties have recently been described (14 - 17).
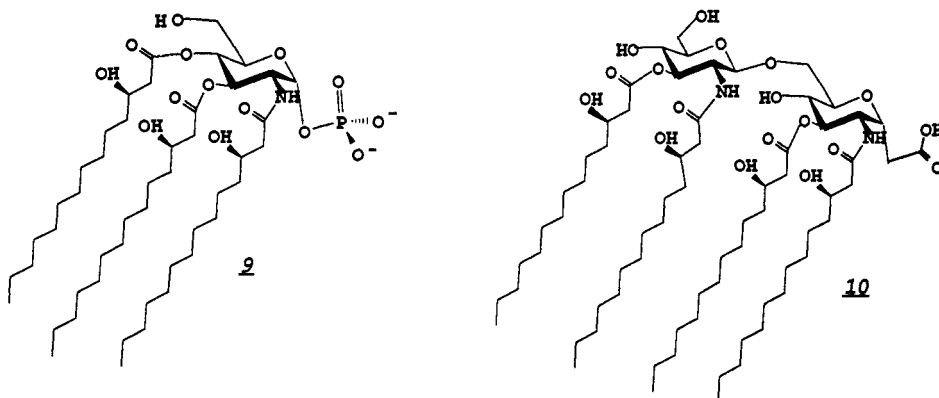
References

1. Gram-negative Septicemia and Septic Shock. In: Infectious Disease Clinics of North America. Vol. 5, Nr. 4, p. 739 - 929, Dec. 1991. Editors: L.S. Young and M.P.Glauser.
2. Bacterial Endotoxin: Recognition and Effector Mechanisms. Endotoxin Series, Vol. 2, p.399 - 412 , Elsevier 1993. Editors: J. Levin, C. Alvin, R. Munford, and P.Stütz.
3. I. Macher. Carbohydr. Res. 162 , 79 - 84, (1987).
4. K. Ikeda, T. Takahashi, C. Shimizu, S. Nakamoto, and K. Achiwa. Chem. Pharm Bull. (Tokyo), 35, 1383 - 1387, (1987).
5. H. Aschauer, A. Grob, J. Hildebrandt, E. Schütze, and P.Stütz. J. Biol. Chem. 265, 9159 - 9164, (1990).
6. K. Briner, and A. Vasella. Helv. Chim Acta 70, 1341, (1987).
7. H. Vyplel, D. Scholz, I. Macher, K. Macher, K. Schindlmaier, and E. Schütze. J. Med. Chem. 34, 2759 - 2767, (1991).
8. Cellular and Molecular Aspects of Endotoxin Reactions, p. 129 - 144, Elsevier 1990. Editors: A. Nowotny, J. Spitzer, E. Ziegler.
9. H. Vyplel, D. Scholz, H. Loibner, M. Kern, K. Bednarik, and Hans Schaller. Tetrahedron Lett. 33, 1261 - 1264, (1992).
10. K. Brozek, C. Bulawa, and C. Raetz. J. Biol. Chem. 262, 5170 - 5179.
11. D. Crowell, M. Anderson, and C. Raetz. J. Bacteriol. 152, 168- .. (1986).
12. D. Scholz, K. Bednarik, G. Ehn, W. Neruda, E. Janzek, H. Loibner, K. Briner, and A. Vasella, J. Med. Chem. 35, 2070 - 2074 (1992).
13. H. Vyplel, D. Scholz, H. Loibner, M.Kern, K. Bednarik, and H. Schaller. Tetrahedron Lett. 33, 1261 - 1264, (1992).
14. C. Lam, E. Schütze, E. Liehl, and P.Stütz. Antimicrob. Agents & Chemotherapy, 35, 506 - 511 (1991).
15. C. Lam, E. Schütze, E. Quakyi, E. Liehl, and P.Stütz. Canadian J. Infect. Diseases 3, Suppl.X, 1 - 8 (1992).
16. E. Quakyi, J. Hildebrandt, G. Bahr, S. Coulibaly, F. Großmüller, A. Rot, E. Liehl, and C. Lam. Submitted to Experimental Hematology 1993.
17. C. Lam, E. Schütze, J. Hildebrandt, H. Aschauer, E. Liehl, I. Macher, and P.Stütz. Antimicrob. Agents & Chemotherapy 35, 500 - 505, (1991).